On the Structure and Function of Amino Acid Transfer Ribonucleic Acid Ligases

Bengt Åkesson

Göteborg 1978

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This thesis is based on the following communications, referred to in the text by their Roman numbers.

I Simultaneous Purification and Some Properties of Aspartate:tRNA Ligase and Seven Other Amino-Acid:tRNA Ligases from Escherichia coli.
(1978) Eur. J. Biochem. 83, 29-36.
Together with L. Lundvik.

II Aminoacyl Adenylate, A Normal Intermediate or a Dead End in Aminoacylation of Transfer Ribonucleic Acid.
(1977) J. Biol. Chem. 252, 1002-1006.
Together with U. Lagerkvist and R. Brändén.

III Allosteric Conformational Changes of Yeast Valyl-tRNA Synthetase on Interaction with $tRNA^{Val}$.
Manuscript (enclosed).
Together with R. Rigler.

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Abbreviations used:

aa, amino acid ;

AMP, adenosine monophosphate ;

A site, aminoacyl site on the ribosome ;

ATP, adenosine triphosphate ;

dATP, deoxyadenosine triphosphate ;

DNA, deoxyribonucleic acid ;

E, enzyme (amino acid:tRNA ligase) ;

E^{Ile}, isoleucine:tRNA ligase ;

G factor, translocase ;

GTP, guanosine triphosphate ;

ligase, amino acid:tRNA ligase ;

mRNA, messenger ribonucleic acid ;

PP_i, inorganic pyrophosphate ;

P site, peptidyl site on the ribosome ;

RNA, ribonucleic acid ;

rRNA, ribosomal ribonucleic acid ;

T factor, elongation factor T ;

tRNA, transfer ribonucleic acid ;

$tRNA^{Ala}$, alanine specific tRNA ;

$tRNA^{Cys}$, cysteine specific tRNA ;

$tRNA^{fMet}$, formylmethionine specific tRNA ;

$tRNA^{Gly}$, glycine specific tRNA ;

$tRNA^{Ile}$, isoleucine specific tRNA ;

$tRNA^{Phe}$, phenylalanine specific tRNA ;

$tRNA^{Ser}$, serine specific tRNA ;

$tRNA^{Val}$, valine specific tRNA ;

Val, valine ;

INTRODUCTION

Since the 19th century it has been known that chromosomes contain two classes of macromolecules namely proteins and nucleic acids. As the nucleic acids contained equal proportions of pentose, phosphoric acid and a heterocyclic base and as there were four different bases believed to be present in equimolar amounts the structure of the nucleic acid was thought to be repetitive and simple ; too simple to contain the genetic information. Until 1944, it was generally assumed that the genetic information was contained in the chromosomal proteins. That year Avery and his group published their discovery that "a nucleic acid of the deoxyribose type is the fundamental unit of the transforming principle of Pneumococcus type III" (1). This conclusion was further supported by the results of Hershey and Chase who showed that deoxyribonucleic acid (DNA) is also the genetic material in bacteriophage T2 (2). In the meantime Chargaff had shown that the four bases in nucleic acids were not present in equimolar amounts and that their relative ratios varied between species (3). These findings indicated a DNA structure complex enough to form the molecular basis for heredity.

To elucidate the three dimensional structure of DNA now became a main objective in biological research and in 1953 Watson and Crick proposed a DNA model (4) based on the high resolution X-ray diffraction patterns obtained by Wilkins' group (5). This model, a double helix where the two strands are held together with hydrogen bonds between complementary purine and pyrimidine bases, immediately suggested a mechanism for how the DNA could duplicate itself. A month after Watson and Crick presented their structural

model (4) they published their hypothesis on replication (6) and they wrote:

> "We imagine that prior to duplication the hydrogen bonds are broken, and the two chains unwind and separate. Each chain then acts as a template for the formation on to itself of a new companion chain, so that eventually we shall have two pairs of chains where we only had one before. Moreover, the sequence of the pairs of bases will have been duplicated exactly".

Kornberg soon found an enzyme, DNA polymerase, that synthesized new DNA from nucleotides in the presence of template and primer (7).

Beadle had earlier formulated the "one gene - one enzyme" hypothesis (8) and now, knowing that DNA is the genetic material and how it replicates, the question arises how the genetic message is translated into the amino acid sequence of a protein? In the mid fifties Crick proposed the existence of an adaptor RNA, transfer ribonucleic acid (tRNA), which could react with a specific amino acid, in a reaction catalyzed by a special enzyme. The tRNA could then combine specifically, by hydrogen bonding, with the nucleic acid template for protein synthesis (9). This hypothesis was substantiated by the results of Hoagland et al. (10) who found that ^{14}C-labeled amino acids were attached to a small ribonucleic acid (RNA) molecule that was able to transfer the amino acids to a protein fraction associated with the ribosomes.

The work of Borsook et al. (11) and Hultin (12) in 1950 as well as that of Hoagland et al. (10) showed, as mentioned above, that incorporation of radioactive amino acids into proteins was associated with the ribosomes in the cytoplasm suggesting the existence of

a carrier of genetic information from the cell nucleus to the ribosomes. In 1961 Jacob and Monod proposed that this carrier should be an RNA molecule which they named messenger RNA (mRNA) (13). They also predicted that its base composition should reflect that of the DNA that specifies it, that it should be heterogeneous in size because the genes vary in length and that it should have a very short life time. Jacob and Monod envisaged the mRNA as part of a sophisticated control system which regulated the expression of the structural genes. To explain the regulatory mechanism they proposed the operon model in which a regulatory gene produces a repressor which interacts with an operator shutting off the structural genes in the operon. Consequently, the structural genes can only be expressed, i.e. give rise to mRNA in a process called transcription which ultimately leads to the formation of the corresponding proteins, when the operator is not blocked by the repressor. This ingenious hypothesis was soon verified experimentally. Brenner et al. discovered the unstable intermediate, the mRNA, carrying information from the genome to the ribosomes (14) while Hall and Spiegelman showed by hybridization that the mRNA was complementary to the transcribed DNA strand (15). An important confirmation of the operon model was the isolation of the lac repressor by Gilbert and Müller-Hill (16). The enzyme responsible for the transcription of DNA, the DNA-dependent RNA-polymerase, was also discovered and characterized at this time (17-19).

Finally, an important question was how the language of nucleotide sequences in the mRNA was translated into amino acid sequences in the proteins. The answer came through the work of Nirenberg (20), Ochoa (21) and Khorana (22) and their co-workers.

Triplets of bases in the mRNA, codons, specify the amino acids to be incorporated into the protein. The codon bases are complementary to a nucleotide sequence in the tRNA, the anticodon, meaning that they can form specific hydrogen bonds with the bases in the anticodon. In this way codon and anticodon can recognize each other and the amino acid attached to the tRNA molecules can consequently be arranged in a genetically determined sequence. These findings have led us to the modern concept of protein biosynthesis described below.

The flow of genetic information in cells is from DNA to RNA to protein. The synthesis of RNA, the transcription, is carried out by an enzyme, RNA polymerase, which uses a gene in a DNA molecule as template. The RNA polymerase recognizes and binds to start signals, promotor sites, on DNA. This leads to local unwinding of the DNA template, thus making it possible to form the first phosphodiester bond in the new RNA chain. The direction of RNA synthesis is from 5′- to 3′- terminal. There are also specific signals on DNA to terminate transcription leading to the release of newly synthesized RNA. Some RNA molecules are then cleaved and chemically modified.

A cell contains three kinds of RNAs, namely mRNA, tRNA and ribosomal RNA (rRNA), all involved in protein synthesis. Messenger RNA is the template for protein synthesis and is very heterogeneous in size since there is an mRNA molecule corresponding to each gene or group of genes on the DNA. Transfer RNA carries the activated amino acids to the ribosome in the cytoplasm for peptide bond formation, in a sequence determined by the mRNA template. There is at least one kind of tRNA for each of the twenty amino acids. Ribosomal RNA together with several different protein molecules

constitute the ribosome. Its precise role in protein synthesis is not yet known.

Procaryote cells regulate their protein synthesis primarily at the level of transcription. The best known case is the lactose operon in Escherichia coli (Fig. 1) where three structural genes are organized as an operon, which is a coordinated unit of gene expression. The operon contains, besides the three structural genes, also an operator and a promotor. Outside the operon,

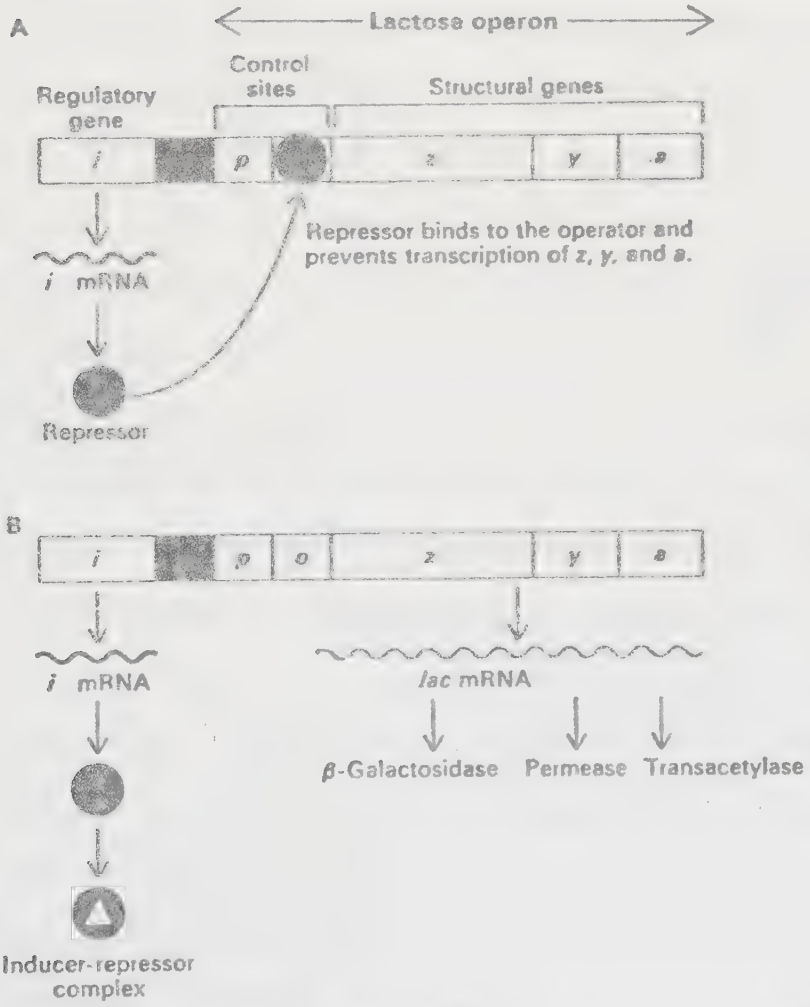


Fig. 1. The lactose operon. Diagram of the lactose operon in the repressed (A) and induced (B) states. (Based on Stryer, L., ref. 23).

there is a regulator gene coding for a protein, the lactose repressor, that can interact with the operator site. The lac-operon contains the structural genes for three enzymes involved in the catabolism of lactose, among them the enzyme β-galaktosidase. The control of gene expression is exactly as proposed by Jacob and Monod (13). In the absence of lactose the repressor, produced by the regulatory gene, binds to the operator and prevents transcription of the structural genes (Fig. 1A). When lactose is present it is converted into allolactose by the small amount of β galaktosidase always present in the cell. The allolactose serves as an inducer and forms a complex with the repressor which can no longer bind to the operator and prevent transcription (Fig. 1B). Instead the RNA polymerase can produce the lactose mRNA which is translated into β -galaktosidase on the surface of the ribosomes.

Translation starts with the formation of an initiation complex involving ribosome, mRNA, formylmethionyl-tRNA^{fMet}, guanosine triphosphate (GTP) as energy source and some additional protein factors, essential for formation of this complex. One of the components, the formylmethionyl-tRNA^{fMet}, like all other aminoacyl-tRNAs needed in translation is synthesized by a specific enzyme, an amino acid:tRNA ligase, in a way that will be discussed in the following chapters. The formylmethionyl-tRNA^{fMet} molecule in the initiation complex occupies the peptidyl site (P site) on the ribosome in such a way that its anticodon pairs with the complementary initiation codon on mRNA. The other site for a tRNA molecule, called the aminoacyl site (A site), is so far empty. In the following step in translation, the elongation (Fig. 2), the next aminoacyl-tRNA is bound to the A site, a

process that requires GTP and a small protein complex, the T factor. In the peptidyl transferase reaction the new peptide bond is formed and the lengthened peptidyl-tRNA is now on the A site and the empty tRNA on the P site. In the translocation

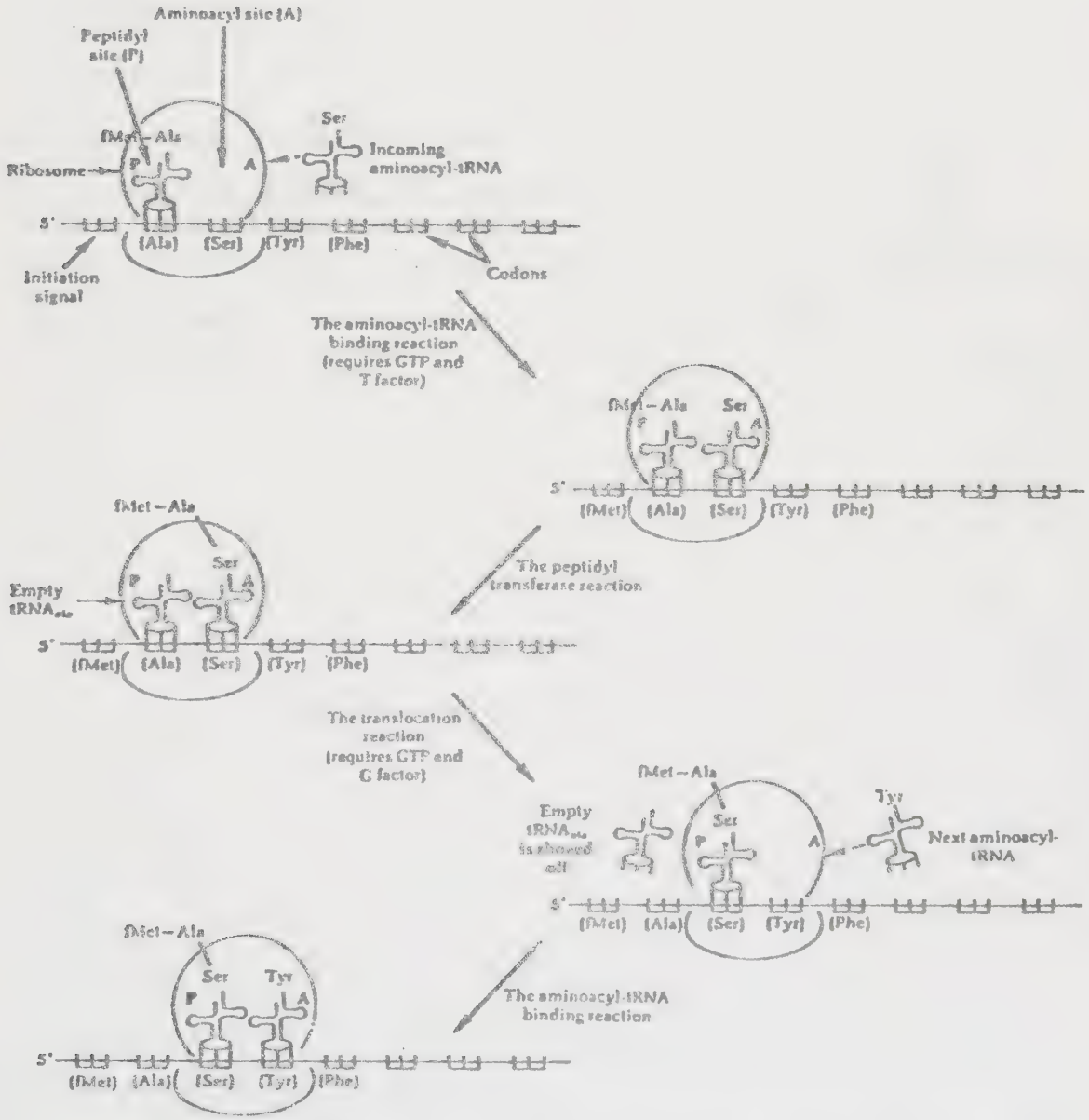


Fig. 2. Steps in elongation. The elongation cycle described is the second one after formation of the initiation complex. (Based on Lehninger, A. L., ref. 24).

reaction, which requires translocase, also called G factor, GTP is hydrolyzed to furnish the energy needed to translocate simultaneously the peptidyl-tRNA from A site to P site and the mRNA by one codon. The cycle is then repeated with the next incoming aminoacyl-tRNA. The termination of translation is determined by special codons that do not correspond to any tRNAs in normal cells. Instead, these stop codons are recognized by release factors that alter the specificity of peptidyltransferase so that water is now the acceptor of the activated peptidyl moiety. The polypeptide chain which leaves the ribosome is sometimes modified in different ways after translation, for example by cleavage, deformylation, disulfide bond formation, hydroxylation or attachment of sugars.

TRANSFER RIBONUCLEIC ACIDS

Crick's adaptor hypothesis in which he predicted the existence of tRNA was soon supported by experimental evidence. Hultin and Beskow showed that labeled amino acids incubated with crude extract from rat liver cells and adenosine triphosphate (ATP) were converted into a stable state where they did not equilibrate with free amino acids (25). Holley showed that this stable intermediate was ribonuclease sensitive (26) and Hoagland et al. demonstrated that amino acids were bound to RNA, i.e. tRNA, independent of each other. On the other hand, binding was proportional to the amount of tRNA suggesting at least one species of tRNA specific for each amino acid (10, 27). The parallel work of Ogata and Nohara also pointed to an amino acid accepting tRNA (28). The adaptor role of the tRNA was definitely proved by Chapeville et al. (29) who showed that when cysteinyl-$tRNA^{Cys}$ was converted into alanyl-$tRNA^{Cys}$ a cell-free polypeptide synthesizing system incorporated alanine as though it was cysteine. This clearly shows that the codon recognition does not depend on the amino acid attached to the tRNA but on the tRNA itself.

Great efforts were now made to obtain tRNA specific for a single amino acid in a pure state. Holley's group introduced counter-current distribution to isolate $tRNA^{Ala}$ from yeast (30) and Tener's group used benzoylated diethyl-amino-ethyl-cellulose (BD-cellulose) to isolate several tRNA species from yeast (31).

In the mid sixties the complete nucleotide sequences of $tRNA^{Ala}$ and $tRNA^{Ser}$ from yeast were presented by the groups of Holley (32) and Zachau (33) respectively. A number of tRNAs

have been investigated since then and to-day almost a hundred sequences are known (34, 35). A comparison of these data shows that tRNA molecules have many common structural features. They contain from 73 to 93 nucleotides corresponding to a molecular weight of about 25.000. They can all be arranged in the two-dimensional cloverleaf model first proposed by Holley (32) where the variation in the number of nucleotides is reflected in the length of the "extra arm" (Fig. 3). They contain between seven and fifteen

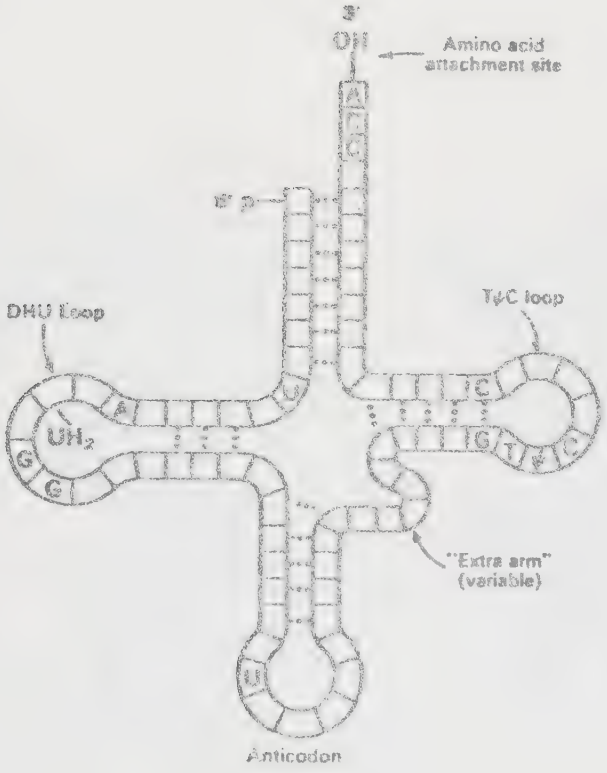


Fig. 3. Common features of tRNA molecules. This schematic cloverleaf structure shows the common features of tRNA molecules. Nucleotides are abbreviated as follows: adenylic acid (A), cytidylic acid (C), guanylic acid (G), uridylic acid (U), dihydrouridylic acid (UH_2) and pseudo-uridylic acid (ψ) (From Stryer, L., ref. 23).

modified bases, the 5'-terminal is always phosphorylated and is usually a guanine, the 3'-end has always the sequence cytosine, cytosine, adenine and there are about ten more invariant bases in the structure, mainly in the different loops. These findings were not unexpected since all tRNA molecules have to interact in a very similar way with ribosomes and mRNA molecules.

In 1974 Rich and Klug with their co-workers independently determined the three-dimensional structure of $tRNA^{Phe}$ from yeast (36, 37). Their X-ray crystallographic studies at a resolution of 3 Å gave a good picture of the molecule (Fig. 4). It is L-shaped

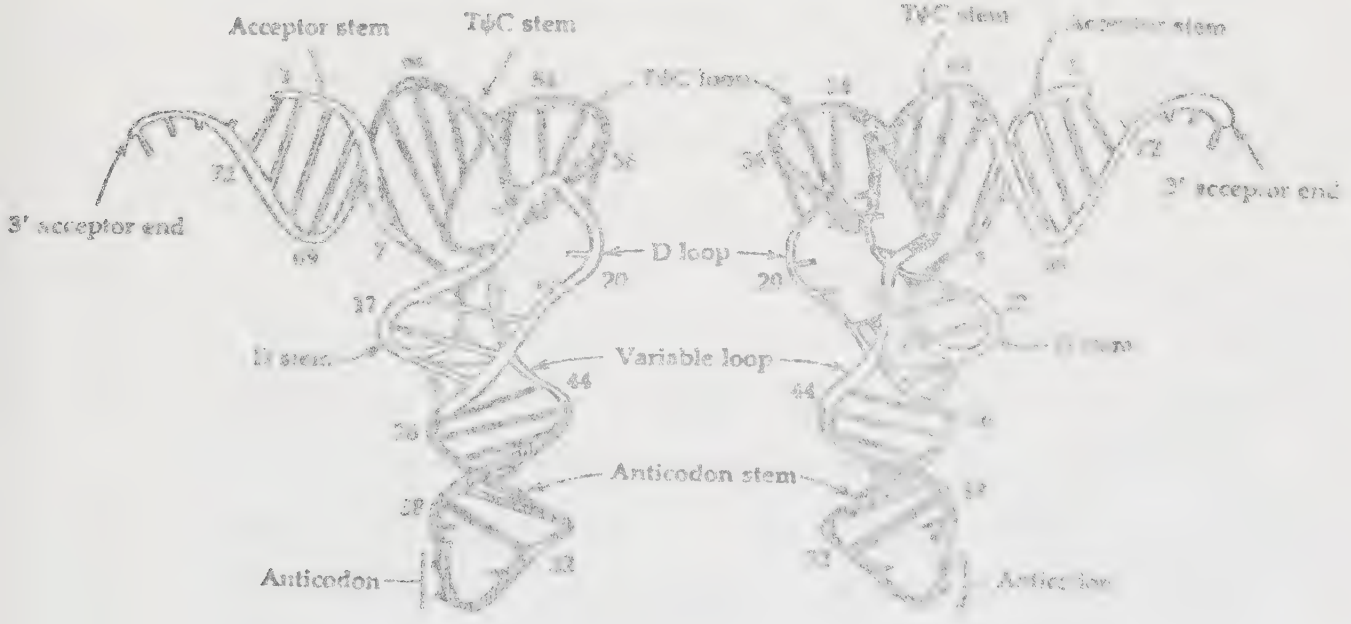


Fig. 4. Transfer RNA structure. Drawing indicating the three-dimensional structure of yeast phenylalanine tRNA. Secondary base-base hydrogen bonding interactions are unshaded or shaded while tertiary ones are black (Based on Quigley, G. J., et al. ref. 38).

with two segments of double helix perpendicular to each other. The 3'-terminal is at one end, weakly interacting with the rest of the molecule, whereas the anticodon loop is at the other end about 80 Å away. The three-dimensional structure confirms the base pairing predicted by the cloverleaf model. Furthermore most of the invariant bases in the different loops participate in tertiary hydrogen bonds to establish the tRNA structure. Should these tertiary hydrogen bonds also prove to be invariant this would indicate a three-dimensional structure that are almost similar for all tRNAs. When earlier observations were re-examined by Rich and Schimmel in the light of the high resolution tRNA structure, they found that apparently disparate findings could be rationalized by postulating that all tRNAs had a common three-dimensional structure and that amino acid:tRNA ligases bound around the side of the tRNA molecule which contains the acceptor stem, dihydrouridine stem, and anticodon (39).

Although great progress has been made the last few years much work still remains to be done in order to determine in detail which parts of the three-dimensional tRNA structure are common to all tRNA molecules and which are unique. This question will obviously be important for our understanding of the structural basis for the specificity of the recognition between an amino acid:tRNA ligase and its tRNA substrate.

AMINO ACID : TRANSFER RIBONUCLEIC ACID LIGASES

As mentioned above Crick postulated a group of enzymes that catalyze the attachment of an amino acid to its cognate tRNA (9) and Borsook et al. (40) and Lipmann (41) argued that protein synthesis was an energy consuming process. This latter suggestion was confirmed when Hoagland and Zamecnik showed that mitochondria could be replaced by ATP (42). They also found a protein factor in rat liver cells that catalyzed an ATP dependent amino acid activation and the exchange of ^{32}P-PP_i with ATP in the presence of amino acids. When they tested several isoelectrically precipitated fractions for activity towards different amino acids the results showed that the different amino acids were activated by separate enzymes (43, 44) as predicted by Crick. At the same time Berg showed a methionine dependent ^{32}P-PP_i-ATP exchange in yeast (45) and DeMoss and Novelli observed the same type of activity in bacteria (46). The final evidence for the proposed amino acid activation mechanism came with the enzymatic synthesis of tryptophanyl adenylate on a preparative scale using a partially purified beef pancreas tryptophan activating enzyme (47-49). These amino acid activating enzymes, now named amino acid:transfer ribonucleic acid ligases, have since been found in all organisms investigated. In the following an enzyme of this type will be denoted by the word "ligase", "aspartate ligase" being aspartate:tRNA ligase, etc.

Function of Ligases

As mentioned above the final evidence of the amino acid activation mechanism came with the enzymatic synthesis of tryptophanyl adenylate (47-49) (Equation 1). This and other similar observations together with the discovery that an isolated enzyme • aminoacyl adenylate complex could transfer its amino acid to tRNA (Equation 2) gave rise to the classical concept of the mechanism involved in aminoacylation of tRNA. In general terms the overall reaction (Equation 3) can be written in two steps as follows.

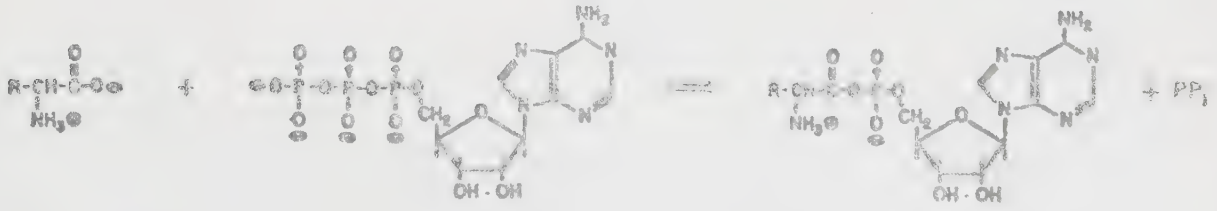
$$E + aa + ATP \xrightleftharpoons{Mg^{2+}} E \cdot aa - AMP + PP_i \qquad \text{(Eq. 1)}$$

$$E \cdot aa - AMP + tRNA \rightleftharpoons aa - tRNA + AMP + E \qquad \text{(Eq. 2)}$$

$$aa + tRNA + ATP \xrightleftharpoons{Mg^{2+}} aa - tRNA + AMP + PP_i \qquad \text{(Eq. 3)}$$

Most ligases are able to form an enzyme bound aminoacyl adenylate complex (E • aa - AMP) in the absence of tRNA (Eq. 1). The exceptions known are ligases specific for arginine (50-52), glutamic acid (53-55), and glutamine (55, 56) regardless of source. The E • aa - AMP complex is stable and can be isolated by gel-filtration (57) or ion exchange chromatography (58). It contains an anhydride bond between the carboxyl group of the amino acid

and the 5′-phosphoryl group of the adenylic acid (Fig. 5). If this complex is a normal intermediate or a dead end in the aminoacylation of tRNA will be further discussed below (II). Amino acid activation is usually assayed by measuring the incorporation of ^{32}P from radioactive PP_i into ATP under conditions of equilibrium (59). The transfer step (Eq. 2) as well as the overall reaction (Eq. 3) can be assayed by measuring the incorporation of amino acid into acid-precipitable aa-tRNA after incubation of the enzyme with radioactive amino acid, ATP, and tRNA (59-61). Discrimination between naturally occurring amino acids is high, although not absolute. For instance, the isoleucine ligase from Escherichia coli catalyzes the activation of both valine (62) and leucine (63). $E^{Ile} \cdot Val\text{-}AMP$ does not, however, transfer the amino acid onto $tRNA^{Ile}$. Instead this complex is hydrolyzed in the presence of the cognate tRNA. The free amino group, but not the carboxyl group, is essential for the activation (64). ATP is the only ribonucleoside triphosphate which is able to supply the energy needed for the activation reaction (65) but dATP, a deoxyribonucleoside triphosphate, can also be used although it is less effective (66).

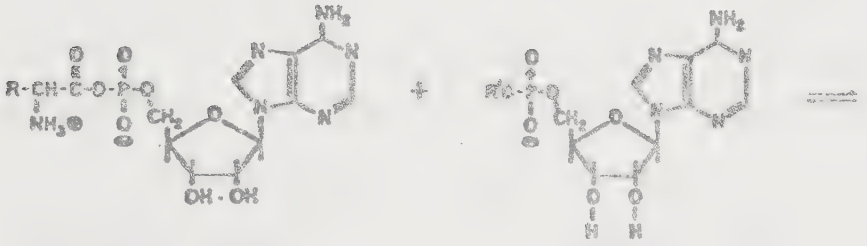


amino acid ATP aminoacyl-AMP

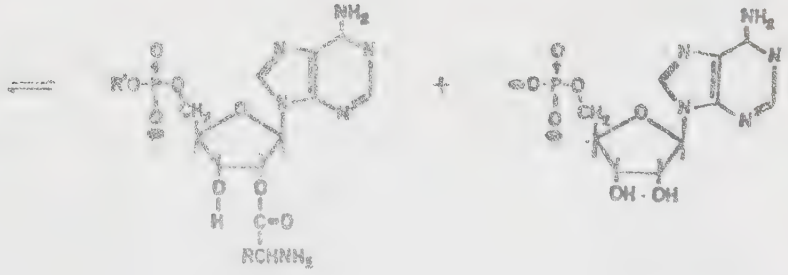
Fig. 5. Formation of aminoacyl adenylate complex (Eq. 1).

R, amino acid side chain.

The product of the transfer reaction (Eq. 2), aminoacyl-tRNA (Fig. 6), has been caracterized as an ester of the amino acid linked to the 2′- or 3′-hydroxyl group of the ribose of the 3′-terminal adenosine in tRNA (67). By modification of this ribose it has been shown that some enzymes aminoacylate at the 2′-hydroxyl group (68) whereas others are specific for the 3′-hydroxyl group and still others are unspecific (69-71). Today it is impossible to see any biological meaning in this variation in specificity among ligases.



aminoacyl-AMP tRNA



aminoacyl-tRNA AMP

Fig. 6. Formation of aminoacyl-tRNA complex (Eq. 2).

R, amino acid side chain ; R′, nucleic acid chain. In this case the amino acid is attached to the 2′-hydroxyl group of the tRNA.

The next step in protein synthesis, catalyzed by elongation factor T, is probably specific for the 2′isomer, while the formation of the peptide bond requires that the amino acid should be in the 3′-position. The rapid spontaneous migration of the amino acid between the two hydroxyl groups makes these different specificities possible.

Ligases can also catalyze the hydrolysis of aminoacyl-tRNA (Equation 4) (72-75).

$$E + aa - tRNA + H_2O \longrightarrow E + aa + tRNA \qquad \text{(Eq. 4)}$$

This reaction is distinct from the reversal of Equation 3 which requires AMP and PP_i. In the first careful studies of this deacylation reaction it was observed that the hydrolysis of the misacylated tRNA was much faster than that of the correctly acylated tRNA when enzyme homologous to the tRNA was used (75-78). This reaction would therefore appear to provide a way to monitor aminoacylated tRNA for incorrect esterification (77). However, the broadness of specificity of the reaction and the fact that in some cases cognate aminoacyl-tRNA can be deacylated at the same rate as the non-cognate, makes it hard to conceive of a useful physiological function for the hydrolysis reaction (78, 79).

After gentle homogenization of eucaryotic cells it was found that all ligases could be isolated in a large lipid containing complex together with tRNA (80), tRNA methylases and other tRNA modifying enzymes (81), elongation factors (82). Ribosomes have also been shown to form complexes with ligases (83). In these complexes the ligases are much more active than in their free state suggesting that the protein synthesizing apparatus may form a highly ordered structure within the cell. This is an almost

untouched area where investigations can lead to interesting discoveries regarding ligases and cell organization.

Several other functions have also been found for the ligases. Specific enzymatic aminoacylation of the 3′-terminal of viral RNA has been demonstrated (84-89) but no biological function of this reaction is known. In gram-positive bacteria it has been shown that aminoacyl-tRNA serves as donor in forming peptide bridges in the mucopeptide of the cell wall (90-96). In some cases (94-96) there is at least one isoaccepting tRNA species that functions uniquely in cell wall synthesis. It is not known if there are unique ligases responsible for charging these species or if the same ligase acts both on tRNA intended for cell wall formation and tRNA intended for protein synthesis.

Aminoacyl tRNA is reported to be the donor also in the biosynthesis of aminoacyl phosphatidylglycerol (97, 98), glycyl lipopolysaccharide (99) and in the addition of certain amino acids to the N-terminus of some proteins (100 and ref. therein). All these ribosome independent processes presumably involve esterification of the tRNAs by ordinary ligases. Ribosome free peptide antibiotic biosynthesis, however, does not require ligases (101, 102).

The isolation of conditional mutants for ligases led to the discovery that these enzymes are, at least indirectly via the formation of aminoacyl tRNA, involved in many cellular regulatory processes, including repression of some amino acid biosynthetic pathways, the control of stable RNA formation, the initiation of DNA formation by reversed transcriptase, the control of protein degradation, and the regulation of the biosynthesis of at least some ligases. A comprehensive discussion on these processes would require a complete monograph and the reader is therefore referred to some recent reviews (103-106).

Structure of Ligases

The amino acid composition of several ligases has been determined (81 and ref. therein, I) but no general conclusion can be drawn from these data. Not even with corresponding enzymes from different sources have any similarities been shown (107-110). So far, the complete primary structure of a ligase has not been determined and our knowledge of the secondary structure is mainly based on a few and not very informative circular dichroism spectras (111-112).

The size and subunit composition of the ligases show considerable differences. The most frequently observed molecular weight is approximately 100,000 but there are enzymes like phenylalanine ligase from yeast and bacteria with a molecular weight of about 270,000 (113-115). The subunit composition can be either a single chain (α), two identical chains (α_2), two non identical chains ($\alpha\beta$), or four chains that are pairwise identical ($\alpha_2\beta_2$) (54, 110, 115, I).

In 1970 Rymo et al. crystallized the lysine ligase from yeast (116, 117) and to date several additional ligases have been obtained in crystalline form (118-121). Low resolution structures for tyrosine ligase from Bacillus stearothermophilus and an enzymatically active fragment of methionine ligase from Escherichia coli have been published (120, 121) and a tyrosine ligase structure at high resolution can be expected in the near future. Although these data have given considerable information on the tertiary and quarternary structures of these enzymes it is of course not possible to draw any general conclusions from them. Hopefully the next few years will yield

high resolution structures of a ligase and its cognate tRNA or even of the enzyme-substrate complex. Such structures would be of the greatest importance for our understanding of enzyme-substrate and protein-nucleic acid recognition in general and ligase-tRNA recognition in special.

There are also some data on the shape and dimension of ligases in solution. Österberg et al. (122) used small-angle X-ray scattering technique to show that the lysine ligase from yeast could be represented by an oblate ellipsoide with the semiaxes 62.7, 50.1 and 23.5 Å. This method was also used in the study of enzyme-substrate complexes that will be commented on below.

Another approach to the investigation of tertiary and quarternary structures is the use of proteolytic enzymes. Treatment of the methionine ligase from Escherichia coli with proteolytic enzymes yielded a single polypeptide chain of molecular weight 64.000 (121, 123) whereas the native enzyme contains two identical subunits with a total molecular weight of 173.000 (124, 125). It is remarkable that, despite the loss of about 200 amino acid residues from each subunit, i.e. nearly 25% of the polypeptide chain, this proteolytic fragment retained full specificity towards methionine and tRNA (123), had identical kinetic parameters in the activation reaction (126), and similar ionic requirements for optimal activity in the ATP exchange reaction and aminoacyl-tRNA formation (127). Another interesting example of proteolytic digestion studies is the leucine ligase from Escherichia coli (128). The native enzyme consisted of a single chain with molecular weight 105.000 whereas the proteolytic modification gave rise to an enzyme with two subunits of 50.000 each plus a small polypeptide of molecular weight 3.000.

This modified enzyme catalyzed the PP_i - ATP exchange, although with less amino acid specificity, but it did not aminoacylate tRNA, in spite of the fact that it bound cognate tRNA. The amino acid specificity was restored if the 3,000 mw polypeptide was added but there was still no aminoacylation. Comparative studies of native and modified forms of these and other enzymes may provide valuable information on their structure-function relationships.

PRESENT INVESTIGATION OF AMINO ACID TRANSFER RIBONUCLEIC ACID LIGASES

The present investigation is part of a long term project where we try to relate the recognition between a ligase and its tRNA substrate to the structure of these molecules. In order to reach this goal it is necessary to obtain several ligases in a highly purified form. The first of these enzymes to be highly purified was the tryptophan ligase from beef pancreas mentioned above (49). Since then many ligases have been purified in several laboratories (129 and ref. therein), including our own (116, 118, 130, 131, I), and from a number of different sources. The procedure worked out for purification of aspartate ligase from Escherichia coli (I) can serve to illustrate the preparative work involved in preparing one of these enzymes. Some structural features of this ligase were also determined (I) and their mechanism of action were studied. As mentioned earlier some ligases can only form the E • aa - AMP complex (Eq. 1) in the presence of their cognate tRNA. This led to the suggestion that aminoacylation of tRNA proceeds directly through a concerted mechanism (132). To elucidate this matter we investigated the early phase of the first catalytic cycle for five highly purified ligases using rapid quenching technique (II).

In the third paper we studied, by means of fluorescence quenching measurements, the interaction between a ligase and its cognate tRNA substrate in solution. Binding parameters as well as conformational changes during catalysis were investigated (III).

Purification and Subunit Structure

The study of aspartate ligase from Escherichia coli was part of a large program and seven other such enzymes were purified simultaneously from the same batch of bacteria (I). The purification procedure is summarized in Table I. The bacterial source was Escherichia coli B grown in a continuous cell culture. The frozen cells were disrupted at -25°C and extracted by stirring in 0.02 M Tris buffer, pH 8.0, at 4°C. Nucleic acids and other acidic components of the crude extract were precipitated with streptomycin sulfate.

Table I : Purification of Aspartate:tRNA ligase

Fraction	Protein	Total activity	Specific activity	Yield
Crude extract	107.000	2.96×10^6	28	100
Streptomycin sup.	64.700	2.56×10^6	40	86
Ammonium sulfate	28.200	1.41×10^6	50	47
DEAE-Sephadex	2.470	0.86×10^6	350	29
Hydroxylapatite	190	0.68×10^6	3.500	23
Affigel	85	0.43×10^6	5.000	14
Gelfiltration, G-100	12	0.18×10^6	15.000	6

The supernatant solution obtained after centrifugation was fractionated by stepwise addition of ammonium sulfate and the fraction containing the major portion of the enzyme activity was chromatographed on DEAE-Sephadex. The following steps were chromatography on hydroxylapatite and Affigel with gel filtration over Sephadex G-100 as the last purification step. The enzyme solution was concentrated by means of negative pressure dialysis and stored at -80°C in 0.1 M phosphate buffer, pH 7.5. Under these conditions about half of the enzyme activity remained after six months. Polyacrylamide gel electrophoresis of this ligase showed one single band, and a single peak was obtained in velocity sedimentation centrifugations. The aspartate ligase had two subunits of the same size and the native enzyme had a molecular weight of 119.000 when measured in velocity sedimentation runs.

A combination of different precipitation and column chromatography procedures like the one just described, have proved to be suitable for purification of most ligases to a stage where they seem to be homogeneous. One must be aware, however, that homogeneous or pure only means that no impurities can be discovered with the different techniques used and that there are no absolute criteria of purity for proteins.

Catalytic Properties

As already mentioned a concerted mechanism has been proposed (132) instead of the classical mechanism of aminoacylation of tRNA (Eq. 1-3). To date there is substantial evidence in support of the classical mechanism. Mg^{2+} has been shown to be essential for equation 1 as well as for equation 3 (133, 134) and there are kinetic data in favor of the classic mechanism (135-138).

In paper II we try to answer the question if aminoacyl adenylate is a normal intermediate or a dead end in the aminoacylation of tRNA. We investigated the early phase of the first catalytic turnover for five highly purified ligases using rapid quenching technique. The experiments were based on three predictions of the stepwise mechanism. The formation of an aminoacyl adenylate by the enzyme must take a certain time and, consequently, the time curve of the aminoacylation reaction could be expected to show a lag before it eventually reached its steady state velocity when the optimum concentration of the intermediate had been reached. Secondly, the lag period should disappear if the enzyme was preincubated with ATP, Mg^{2+} and amino acid before it was blended with the tRNA. Finally, under conditions when the enzyme exclusively used a preformed aminoacyl adenylate for the esterification of tRNA, it should do so at a rate which is at least the same as the steady state rate of the overall reaction.

Of the five ligases investigated, four showed a lag in their time curves for the esterification of tRNA during the early phase of the first catalytic turnover. In each case the turnover number, calculated from the steady state velocity reached after the lag period,

was of the same order of magnitude as that determined for the enzyme under ordinary assay conditions. The lag of the time curves could always be abolished by preincubating the enzyme with ATP, Mg^{2+}, and amino acid and then starting the reaction by mixing with tRNA. The steady state velocities observed in the preincubation experiments were the same as those obtained after the lag period in experiments without preincubation. The only exception was the lysine enzyme from yeast where the steady state velocity in the preincubation experiment was approximately half of that in the experiment without preincubation. The valine enzyme did not show a lag in its time curve, but the steady state velocity observed in an experiment with a preformed valyl · adenylate complex was the same as that obtained in a normal experiment without preincubation. The results of the experiments on preferential utilization of aminoacyl adenylate clearly show that all five enzymes will utilize a preformed, enzyme-bound aminoacyl adenylate rather than free amino acid in the esterification of tRNA.

One of the reasons for postulating a concerted mechanism has been the alleged slowness of the esterification of tRNA by an aminoacyl adenylate · enzyme complex. However, our results with the lysine, serine, and phenylalanine ligases from Escherichia coli show that these enzymes give the same steady state velocity in an experiment where the enzyme uses almost exclusively the preformed aminoacyl adenylate for the esterification of tRNA as under conditions when the reaction was started by mixing the enzyme with its substrates without preincubation. With the lysine enzyme from yeast there is a difference in rate in these experiments but the steady state velocities observed are nevertheless of the same order of magnitude. In any

case, it cannot be maintained that the velocity when tRNA is esterified by a preformed aminoacyl adenylate · enzyme complex is too slow to be compatible with a reaction mechanism involving this compound as an intermediate. The initially higher velocities in some of the experiments with a preformed aminoacyl adenylate cannot be explained by the simple mechanism given in Equations 1 to 3. A possible explanation might be that these enzyme preparations contain a small number of enzyme molecules with a considerably higher turnover number than the rest. However this may be, our main observations - the lag period and its disappearance when the enzyme was allowed to form an aminoacyl · adenylate complex before the start of the esterification reaction as well as the rate of aminoacylation of tRNA using such a complex - are consistent with a reaction mechanism which involves an enzyme-bound aminoacyl adenylate as an obligatory intermediate in the esterification of tRNA by these enzymes. At the same time it should be pointed out that alternative explanations are by no means excluded. For instance, the reaction could proceed by a concerted mechanism and still show a lag if the binding of ATP, Mg^{2+}, and amino acid were accompanied by a time-consuming conformational change which was necessary for full catalytic activity of the enzyme.

The order of binding of reactants has been examined for a number of these enzymes using kinetic methods. In some cases ATP was shown to bind first, but in other cases binding was random, and sometimes, a mixed type of reaction was obtained (139 and ref. therein). This may just reflect the inadequacy of the experimental approaches used but it could also be due to real differences in the catalytic mechanisms of different ligases.

The absolute dependence of the ATP-PP_i exchange reaction catalyzed by arginine (50-52), glutamic acid (53-55) and glutamine (55, 56) ligases on tRNA is also interesting from an evolutionary point of view. So far, regardless of source, all ligases specific for these amino acids have been found to be dependent on tRNA for the exchange reaction. It is surprising that this effect, apparently correlated with the structure of the amino acid recognized, has been retained through evolution although the subunit structure of the enzymes has diverged. For some unknown reason, as far as these ligases are concerned, there must be a strong evolutionary selection for this mechanism.

The dependence of the ATP-PP_i exchange reaction on tRNA is the most obvious example of an allosteric effect of one ligase substrate on the binding of another. There have been some studies on structural changes of the ligases during their catalytic cycle. An example of this is the work by Ohta et al. (111) who used circular dichroism measurements to show a change in the helical content of tyrosine ligase from yeast on binding its cognate tRNA.

Another approach to the study of conformational rearrangements in a cognate ligase-tRNA complex is the use of fluorescence quenching technique. For the serine (140, 141) and phenylalanine (142) ligases from yeast the number of binding sites for tRNA has been determined and evidence has been obtained for structural changes in the cognate ligase-tRNA complexes. In paper III we have used this technique to study the interaction between $tRNA^{Val}$ and valine ligase from Saccharomyces cerevisiae. For the determination of the number of binding sites, two methods have been used to observe complex formation between tRNA and ligase. One method involved measuring

the tRNA induced quenching of the tryptophan fluorescence of valine ligase when the concentration of $tRNA^{Val}$ was increased. The other approach was to determine the rotational tumbling of ethidium labeled $tRNA^{Val}$ as the concentration of valine ligase was increased. By observing the change in rotational tumbling of $tRNA^{Val}$ when bound to the much bigger ligase the binding process can be followed directly. For this purpose the tRNA must have a fluorescent group and it was therefore labeled with ethidium bromide. Both methods yielded consistent results: One binding site for $tRNA^{Val}$ was found.

In order to obtain more detailed information about the reaction mechanism, kinetic studies were performed by observing the relaxation of the interaction between $tRNA^{Val}$ and valine ligase into a new equilibrium state after a temperature jump. When observing the fluorescence intensity of valine ligase, two relaxation processes could be observed. To assess the nature of these two processes the dependence of their life-time constants on the concentration of $tRNA^{Val}$ and valine ligase was investigated. It was found that the life-time constants were increased as the free concentration of $tRNA^{Val}$ and valine ligase was increased. It is evident from this behaviour that these two relaxation times are due to conformational changes of the valine ligase-$tRNA^{Val}$ complex, which follow the association-dissociation step of the tRNA synthetase reaction. The slower of these transitions occurs with a time constant compatible with the turnover number of the enzyme and probably constitutes one of the rate limiting steps for the catalytic activity of the enzyme. With ethidium labeled $tRNA^{Val}$ as the fluorescence marker the same two relaxation processes and similar time ranges

were observed as in the experiments where the intrinsic tryptophan fluorescence of the enzyme was measured.

The results are consistent with the presence of at least three conformational states of the enzyme in a dynamic equilibrium, the rates of which are determined by the presence of $tRNA^{Val}$. This is the first time that conformational transitions of a ligase are observed in a time range comparable to that of the aminoacylation of tRNA. Data obtained from ethidium bromide-$tRNA^{Val}$ complexes suggest that $tRNA^{Val}$ participates in the conformational transitions of valine ligase through changes of its own conformation.

Another method to investigate the ligase-tRNA complex is the small-angle X-ray scattering technique used by Österberg et al. (143). Their results from the lysine yeast system show that the dominating complex in solution includes two enzyme molecules and one tRNA molecule. We extended these investigations to three additional ligases representing different groups of subunit structure: The lysine ligase from Eschirichia coli, which is similar to the corresponding yeast enzyme in having two identical subunits (α_2), the valine enzyme from yeast with one subunit consisting of two identical domains (pseudo-α_2) and the phenylalanine ligase from Escherichia coli with four pairwise identical subunits ($\alpha_2\beta_2$). Our results showed that both the valine and the two lysine ligases formed dimeric (pseudo-α_4 and α_4 respectively) complexes with their cognate tRNAs, whereas the phenylalanine ligase retained its native form with four subunits when binding the tRNA (Österberg, R., Sjöberg, B., Ligaarden, R., and Åhsson, B., unpublished results).

CONCLUDING REMARKS

The amino acid:tRNA ligases play a key role in protein synthesis. There is one specific enzyme and at least one, in most cases several, tRNA for each amino acid. The very high fidelity of the translation of genetic information from nucleic acid to protein requires that the ligases select their tRNA and amino acid substrates with great specificity in order to esterify the correct amino acid to its cognate tRNA. The ligases are also, apart from their role in protein synthesis, very interesting enzymes because of their ability to serve as model systems for studies of enzyme-substrate and protein-nucleic acid interactions in general.

In the present investigation we have worked out a method for purification of eight ligases from the same batch of Escherichia coli. One of these enzymes, the aspartate ligase, has not been obtained in a highly purified form before and was therefore characterized in terms of its molecular parameters. We have also investigated the early phase of the first catalytic turnover for five highly purified ligases using rapid quenching technique. We demonstrated that four of the five ligases, chosen at random, showed a lag period in the time curve of the aminoacylation reaction (Eq. 3) and that this lag could be abolished by preincubating the enzymes with ATP, Mg^{2+}, and amino acid (Eq. 1). In all cases the steady state rate of transfer from the preformed aminoacyl-adenylate complex to tRNA (Eq. 2) was approximately the same as that of the overall reaction (Eq. 3). These results are consistent with the classical, stepwise mechanism (Eq. 1-3). Finally the interaction between valine ligase from Saccharomyces cerevisiae and its cognate tRNA was studied

by means of fluorescence quenching measurements. These experiments showed that the ligase has one binding site for $tRNA^{Val}$ and that there are at least three conformational states of the enzyme in a dynamic equilibrium, the rates of which are determined by the presence of $tRNA^{Val}$. It would seem that $tRNA^{Val}$ participates in the conformational transitions of valine ligase by changing its own conformation.

During the last few years great progress has been made in the elucidation of the three-dimensional structure of both ligases and tRNA and it would seem that we will soon be able to build our theories of the recognition between ligase and tRNA on the firm foundation of detailed structural information. A low resolution structure of the tyrosine enzyme from Bacillus stearothermophilus has been determined and a high resolution structure can be expected in the near future. The same is true for an enzymatically active proteolytic fragment of the methionine ligase from Escherichia coli. Our understanding of the structure of $tRNA^{Phe}$ from yeast has reached a level of considerable precision and refinement and structural studies of $tRNA^{fMet}$ from both Escherichia coli and yeast and $tRNA^{Gly}$ from Escherichia coli are now well advanced. A full understanding of the structural basis for the interaction between tRNA and ligase will probably require co-crystallization of enzyme and substrate tRNA in a form amenable for crystallographic analysis. Although such crystals have not yet been reported there are hopeful indications that this important break through may come in the near future.

The pre-eminence of crystallographic techniques in structural analysis should not make us lose sight of the fact that crystallographic

structures must, as far as possible, be substantiated by methods which can be applied in solution where, after all, most biochemical processes take place. Techniques like NMR, fluorescence quenching and X-ray small angle scattering will obviously play an important role in this respect.

There are many other lines of investigation in the field of tRNA and ligase research which are likely to yield interesting results in the future. We have known for some time of indications that tRNAs and ligases in eucaryotic cells form aggregates, which can be isolated and analyzed, and it is tempting to envisage the many different molecules that make up the translational apparatus as buildingstones in a biochemical architecture of great structural complexity and functional sophistication. Other important problems, where much still remains to be done, concern the processing of the primary transcript of the tRNA genes by specific nucleases and base modifying enzymes and the role of tRNA in cellular regulatory functions.

In any event, it is obvious that research in this field has not lost its momentum over the years and it is, in fact, even more exciting today than it was more than twenty years ago when ligases and tRNA were first discovered.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to

Professor Ulf Lagerkvist for his never failing support and valuable criticism.

Mrs Anne-Marie von Essen, Mrs Birgitta Grundström, Mrs Tullan Andersson and Miss Marianne Wedin for their skilful technical assistance.

Mrs Barbro Nyberg for her careful typing of the manuscripts.

My wife Ingrid for her selfless support and great patience and my sons for their very special and encouraging interest in the laboratory work. To these three this thesis is dedicated.

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ALLOSTERIC CONFORMATIONAL CHANGES OF YEAST VALYL-tRNA SYNTHETASE ON INTERACTION WITH $tRNA^{Val}$

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SUMMARY

The interaction with $tRNA^{Val}$ and valyl-tRNA synthetase from yeast has been studied by measuring the fluorescence quenching of the tryptophan residues of valyl-tRNA synthetase induced by $tRNA^{Val}$ as well as the increase in the rotational tumbling time of $tRNA^{Val}$-ethidium bromide complexes induced by the binding to valyl-tRNA synthetase. Both methods yielded consistent results: one binding site for $tRNA^{Val}$, a binding constant of $3 \times 10^6 M^{-1}$ at 21^oC, a ΔH of -2.6 Kcal/mole and a ΔS of +20.4 cal/mole^oK was found.

Relaxation kinetic experiments indicate the presence of two conformational transitions of the valyl-tRNA synthetase in the time range of 20 msec and 130 msec. The time constants for the conformational transitions are increased to 50 msec and 500 msec if valyl-tRNA synthetase is in complex with tRNA. The rate constant of the slower transition ($2\ sec^{-1}$) is compatible with the experimentally observed turnover number and probably constitutes one of the rate limiting steps of the tRNA charging reaction. The results are consistent with the presence of at least three conformational states of the enzyme in a dynamic equilibrium, the rates of which are determined by the presence of $tRNA^{Val}$. Data obtained from ethidium bromide-$tRNA^{Val}$ complexes suggest that $tRNA^{Val}$ participates in the conformational transitions of valyl-tRNA synthetase by changes of its own conformation.

INTRODUCTION

The selection of cognate tRNA by aminoacyl-tRNA synthetases is the first step in the reaction sequence leading to the translation of the genetic message into a peptide sequence. Since the selection process at least in part must be related to the manner in which tRNAs interact with aminoacyl-tRNA synthetases, the interaction of a variety of aminoacyl-tRNA synthetases with cognate and non cognate tRNA has been investigated. For two yeast enzymes, namely seryl-tRNA synthetase (1,2) and phenylalanyl-tRNA synthetases (3) evidence was given for a conformational rearrangement of the cognate tRNA synthetase complex occuring within a 1-50 milliseconds after the first binding step. We now present evidence for the presence of allosteric conformational transitions in the time range of 50 msec and of 500 msec in valyl-tRNA synthetase induced by the interaction with $tRNA^{Val}$. The slower transition occurs with a time constant compatible with the turnover number of the enzyme and probably constitutes one of the rate limiting steps for the catalytic activity of the enzyme.

MATERIALS AND METHODS

Materials

$tRNA^{Val}_{II}$, and valyl-tRNA synthetase were prepared as described previously (4,7). The $tRNA^{Val}$ accepted 1.2 nanomole valine/A_{260} in the standard aminoacylation assay. Concentrations of $tRNA^{Val}$ were determined by absorbance measurements using an absorption coefficient of 625 mM^{-1} cm^{-1}. The concentration of the valyl-tRNA synthetase was determined by protein determination according to Lowry or Kjeldahl as described in (17).

C^{14} labelled-L-valine was purchased from the Radiochemical Center, Amersham, England, ATP from Boehringer, Mannhein, Germany. Ethidium bromide was a gift of Boots

Pure Drug co ltd, Nottingham, England. For all experiments a potassium phosphate buffer containing 0.1 M ptassium-phosphate, 5 mM $MgCl_2$, 0.2 mM dithiothreitol pH 7.0 was used.

Methods

Enzyme and tRNA assays: Assays for enzyme acitivities and the tRNAs ability to form aminoacyl-tRNA were performed as described in (4) and (8). Radioactivity measurements were performed in a Packard-liquid scintillation spectrometer, model 2405 using Insta-gel (Packard Instruments Company) diluted with 0.6 volumes of Xylene as a scintillation fluid.

Fluorescence titrations: The titrations were performed in a fluorescence spectrometer described in (9) permitting the simultaneous registration of total fluorescence intensity, degree of polarization and anisotropy as well as of absorbance. The fluorescence of valyl-tRNA synthetase and ethidium bromide were excited at 280 nm and 456 nm respectively. The fluorescence emission was measured after passing cut-off filters (Schott u. Gen.) WG 305 (valyl-tRNA synthetase), OG 590 (ethidium bromide). For reabsorption of light asciting the valyl-tRNA synthetase fluorescence by $tRNA^{Val}$ a correction procedure as described in (10) was used.

The polarization (p) and anisotropy (r) of fluorescence were determined by measuring the light components vibrating parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the direction of polarization of the exciting light beam. Correction for depolarization of the optical system and calculation of the degree of saturation during a titration were performed as described in (11,12).

The titrations were performed such that all concentrations except the varied one were kept constant. The enzyme acitivity was checked before and after the titration and

kinetic experiments. The specific activities at the end of the experiments was found to be higher than 90% of the starting value.

Kinetic measurements

Relaxation kinetic experiments were performed in a temperature jump apparatus as described in (9). The equilibrium between tRNA and synthetase was perturbed by a rapid temperature jump (rise time 1 μsec) between 5^{o}-7^{o}C and the fluorescence intensity (I) was recorded as a function of time.

RESULTS

1. Determination of binding parameters by titration experiments

For the determination of the number of binding sites and the temperature dependence of the binding constant, two approaches have been used to observe complex formation between tRNA and synthetase, (i) registration of the tRNA induced quenching of the tryptophan fluorescence of valyl-tRNA synthetase when the concentration of $tRNA^{Val}$ is increased, (ii) registration of the rotational tumbling of ethidium labelled $tRNA^{Val}$ as the concentration of valyl-tRNA synthetase is increased.

a) tRNA induced quenching of the enzyme fluorescence

When saturating amounts of $tRNA^{Val}$ were added to valyl-tRNA synthetase the fluorescence intensity of the enzyme due to tryptophan was reduced to about one half (Fig. 1a). From the concentration dependent change of the enzyme fluorescence, the apparent binding constant and number of binding sites can be calculated (13,11). Analysis of the data according to Scatchard (Fig. 1b) yield a stoichiometry of 1.2 moles $tRNA^{Val}$ per 1 mole of valyl-tRNA synthetase and an equilibrium constant of $K=2.9\times10^{6}M^{-1}$ at 21.5^{o}K (TABLE I).

b) Enzyme induced change of the rotational tumbling of tRNA

By observing the change in rotational tumbling of $tRNA^{Val}$ (Mw~26.000) when bound to the much bigger synthetase (Mw 122.000 (17)) the binding process can be followed directly. For this purpose the tRNA must have a fluorescent group. From its polarized emission ($I_{||}$, $I_{\perp}$) the rotational tumbling times τ_ρ of the carrier tRNA can be determined. Usually the anisotropy of fluorescence

$$r = \frac{I_{||}-I_{\perp}}{I_{||}+2I_{\perp}}$$ is determined and this is related to τ_ρ as:

$$r = \frac{r_o}{1+\tau/\tau_\rho}$$

with τ= lifetime of fluorescence label. This relation implies that r increases with τ_ρ to a limiting value r_o which means that the carrier is virutally immobile in the time window given by τ.

For labelling the $tRNA^{Val}$ the reversible association with ethidium bromide (EB) was used. For $tRNA^{Phe}$ (14,15) and $tRNA^{Ser}$ (15) from yeast one strong binding site was found for the binding of ethidium bromide, the fluorescence of which is strongly enhanced on binding to tRNA. In order to determine the conditions under which $tRNA^{Val}$ binds ethidium bromide, the binding constant and the number of binding sites were determined by titration experiments (Fig. 2a). They show a 36 fold increase of the fluorescence intensity of EB as well as an increase of r from 0.029 to 0.168 due to the immobilization of EB by the tRNA. From the titration curves (Fig. 2b) one strong binding site (n=1.4) with a K of $8.6 \times 10^6 M^{-1}$ was derived. This implies that from a concentration of ~1 µM (and upwards) of both $tRNA^{Val}$ and EB each tRNA molecule is labelled by one EB molecule.

The binding of EB-$tRNA^{Val}$ to valyl-tRNA synthetase was now analyzed by following r of EB-$tRNA^{Val}$ as the

concentration of valyl-tRNA synthetase was increased (Fig. 3a). The titration curves show an increase of r from 0.167 to 0.259 under saturating conditions, while the fluorescence intensity of EB-tRNA^{Val} remains practically unchanged. With the lifetime τ constant, which follows from the unchanged fluorescence intensity of EB-tRNA^{Val}, the increase in r can be attributed solely to the immobilization of EB-tRNA^{Val} by the enzyme. Analysis (Fig. 3b) of the binding data from the concentration dependence of r (11,12) arrives at the same result as the experiments in which the fluorescence quenching of the enzyme was monitored, i.e. n was found to be 1.3 and $K=3.2\times10^{6}$ at 21.3°C. From the temperature dependence of K a reaction enthalpy ΔH of -2.6 kcal/mole and a reaction entropy of 20.4 cal/mole °K was derived. No temperature dependence of n (1.1-1.3) was observed.

2. Kinetic experiments

In order to obtain more detailed information about the reaction mechanism, kinetic studies were performed by observing the relaxation of the interaction between tRNA^{Val} and valyl-tRNA synthetase into a new equilibrium state after a temperature jump. When observing the fluorescence intensity of valyl-tRNA synthetase, two relaxation processes with opposite signs of their amplitude could be observed (Fig. 4a, 4b). The first one has a time constant (τ_1) of 28 msec, the second (τ_2) of 460 msec. To assess the nature of these two processes the dependence of τ_1 and τ_2 on the concentration of tRNA^{Val} and valyl-tRNA synthetase was investigated (Fig. 6a, 6b). In these experiments both tRNA^{Val} and valyl-tRNA synthetase were applied in equeal amounts with increasing concentrations. It was found that the reciprocal value of τ_1 ($1/\tau_1$) and of τ_2 ($1/\tau_2$) was decreased as the free concentration of tRNA^{Val} and valyl-tRNA synthetase was increased.

It is evident from this behaviour as will be discussed later that these two relaxation times are due to conformational changes of the $tRNA^{Val}$-valyl-tRNA synthetase complex, which follow the association-dissociation step of the tRNA synthetase reaction. From the relaxation kinetic data obtained on seryl-tRNA synthetase (1,2) and phenylalanyl-tRNA synthetase (3) we expect the relaxation under the ionic conditions and concentrations used to occur in a time range between 1-10 msec. Such a relaxation process could be clearly observed at the higher concentrations applied (10 µM) occurring with a time consant τ_o=5 msec (Fig. 4c).

Since ethidium reversibly bound (16) as well as covalently linked (17,18) has proven to be a sensitive probe for conformation changes in tRNA, kinetic experiments were performed to see if the relaxation processes when the signal from the synthetase is observed, can also be detected when the signal from the EB labelled $tRNA^{Val}$ is measured. Also in this case two relaxation processes in similar time ranges as τ_1 and τ_2 are found. For the conditions in Figs. 5a and 5b the corresponding relaxation times are: τ_1=19 msec, τ_2=200 msec.
In the concentration range investigated, $1/\tau_1$ and $1/\tau_2$ decreases when the concentration of $tRNA^{Val}$ and VRS were increased in equimolar amounts with the EB concentration fixed at 5×10^{-7}M (Figs. 6a and 6b).

DISCUSSION

From the measurements of the yeast valyl-tRNA synthetase a simple picture turns up which is in line with what has been found for other synthetases: the 1:1 stoichiometry for the interaction with $tRNA^{Val}$ corresponds to the values given for the E.coli enzyme (13) which is of the same size as the yeast enzyme. The moderate (negative) values of ΔH and positive values for ΔS are similar to that what has been reported for a variety of synthetase-tRNA interactions (1,19,20).

The estimated value of n which is greater than one probably is caused by uncertainties in determination of the amounts of tRNA and synthetase which lead to the formation of the complex. This applies particularly for the tRNA, the concentration of which has been determined from the optical density and no consideration was given to the possibility that part of the tRNA could have a structure which is unable to bind to the synthetase. If one corrects for the fact that the $tRNA^{Val}$ used accepted 1.2 nmole valine/A_{200} instead of 1.7 nmole if all tRNA molecules could be aminoacylated, the stoichiometry is found to be very close to one and agrees with the values found for the stoichiometry of the valyl-tRNA synthetase-ATP and the valyl-tRNA synthetase-valine complex respectively (n=1, (7)).

The identical values for the binding parameters obtained from titrations using EB labelled and unlabelled tRNA show that ethidium does not influence the interaction between $tRNA^{Val}$ and valyl-tRNA synthetase. The observation of one strong binding site at least for yeast $tRNA^{Phe}$ can now be based on a structural basis. The position of one ethidium molecule in the crystal structure of $tRNA^{Phe}$ has been located near U_8 (21). Since complex formation of EB-$tRNA^{Val}$ with valyl-tRNA synthetase does not change the fluorescence of EB which is dependent on its interaction with tRNA, it can be concluded that the strongly bound EB is not displaced from the tRNA by the synthetase. Even a transfer of EB from tRNA to the synthetase can be

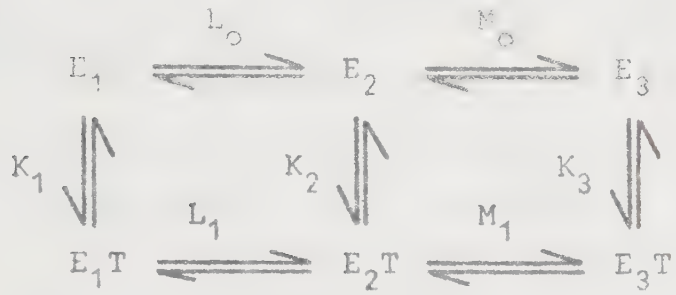
excluded. Binding of EB to valyl-tRNA synthetase as seen from the increase in anisotropy (Fig. 3a) is not accompanied by an enhancement of EB-fluorescence. Thus a drop in the fluorescence intensity of EB-$tRNA^{Val}$ is to be expected in the case of a transfer. This is not observed.

The adduct labelling of $tRNA^{Val}$ with EB is thus a convenient way to investigate the interaction of $tRNA^{Val}$ with its synthetase in analogy to what has been found for the interaction of yeast $tRNA^{Ser}$ with its cognate synthetase (12,15).

Interesting details of the binding process are revealed by the kinetic experiments. Both relaxation times, when the valyl-tRNA synthetase fluorescence is observed are slowed down when the concentration of tRNA and valyl-tRNA synthetase are increased which is opposite to what would be expected from a simple association-dissociation reaction. In this case, because of the increased probability of reassociation at higher concentrations, τ would become shorter. From this particular behaviour of $1/\tau_1$ and $1/\tau_2$ (22,23) two conclusions can be drawn: (i) the synthetase exists in more than one conformation in solution which are in a dynamic equilibrium, (ii) the rate of equilibration is slowed down to an assymptotic value when the enzyme forms a complex with tRNA. The situation is most evident for the slower relaxation time, while the change of the faster relaxation time is at the limit of significance.

The notion of observing two conformational transitions of the enzyme is also evident from the fact that both relaxation times can be observed with the enzyme alone. Two relaxation times τ_1=21 msec and τ_2=10 msec were found (Fig. 6a and 6b) which did not change when the valyl-tRNA synthetase concentration was varied from 0.5×10^{-7}M to 2×10^{-6}M.

The presence of two conformational transitions imply the existence of three structural states of the valyl-tRNA synthetase. The data are thus consistent with a reaction scheme of three enzyme conformations E_1, E_2 and E_3 in a dynamic equilibrium, with transition rates which are slowed down by interaction with tRNA (T).



(I)

The dependence of the relaxation time on the concentration of reactants for this scheme can be calculated for the case that transition $E_1 \rightleftharpoons E_2$ isomerizes rapidly compared to $E_2 \rightleftharpoons E_3$, i.e. $\tau_1 \ll \tau_2$, what is observed experimentally. Furthermore we assume that the binding step equilibrates rapidly compared to the isomerization step.

For the actual experimental situation we obtain for $1/\tau_1$ and $1/\tau_2$ with the definition:

$$\frac{E_2}{E_1} = L_o = \frac{l_o}{l_{-o}} \quad , \quad \frac{E_3}{E_2} = \frac{m_o}{m_{-o}}$$

$$\frac{E_2T}{E_1T} = L_1 = \frac{l_1}{l_{-1}} \text{ and } \frac{E_3T}{E_2T} = \frac{m_1}{m_{-1}}$$

$$\frac{1}{\tau_1} = \frac{l_o + l_1 K_1 TX}{1 + K_1 TX} + \frac{l_{-o} + l_{-1} K_2 TY}{1 + K_2 TY}$$

and

$$\frac{1}{\tau_1} = \frac{L_o(m_o + m_1 K_2 TY)}{L_o(1 + K_2 TY) + (1 + K_1 TY)} + \frac{m_{-o} + m_{-1} K_3 TZ}{1 + K_3 TZ}$$

where X, Y, and Z are functions of T, E, K_1, K_2, K_3, L_o and M_o. They consider the fact that in the present experimental situation the concentration of free tRNA T is the same as the free concentration of the synthetase E and the binding of T to E_1 is influenced by the simultaneuous binding of T to the two other conformations E_1 and E_2. For T>>E, this coupling of reactions is abolished and the coupling factors X, Y, and Z approach unity.

We now see that for low concentrations of E and T, $1/\tau_1$ approaches l_o+l_{-o} and becomes

$$\frac{1}{\tau_1} = l_o + l_{-o} \quad \text{if} \quad T = 0.$$

With increasing concentrations of E and T, $1/\tau_1$ reaches a new value

$$\frac{1}{\tau_1} = l_1 + l_{-1}$$

i.e. the sum of the rate constants for isomerization of the synthetase-tRNA complex.
For $1/\tau_2$ the situation is analogous:

$$\frac{1}{\tau_1} = \frac{L_o m_o}{L_o+1} + m_{-o} \quad \text{for} \quad T = 0.$$

and

$$\frac{1}{\tau_1} = \frac{L_o m_1 K_2 Y}{L_o K_2 Y + K_1 X} + m_{-1}.$$

Reexaminating now the meaning of the equilibrium constant determined previously, it is evident that this constant must incorporate all equilibrium parameters of the reaction scheme (I). It can be shown that the binding constant as determined from the Scatchard plot (K^*) is related to K_1, K_2, K_3, L_o and M_o by:

$$K^* = \frac{K_1+L_o(K_2+M_oK_3)}{1+L_o(1+M_o)}$$

Thus the related thermodynamic parameters ΔH and ΔS are constituted from the ΔH and ΔS values of all equilibrium constants.

The kinetic picture given here is characteristic for what has been found for allosteric enzymes. Here the shift from the T to the R-form is caused by a higher affinity of the substrate for the R-form which slows down the transition form R to T but leaves the transition form T to R unaffected. The classical example is glyceraldehyde-3-phosphate dehydrogenase (24). Here the allosteric transitions are potentiated by the cooperative interaction with the cosubstrate NAD.

With a few approximations we can see that the situation for valyl-tRNA synthetase resembles the case of GAPDH. Let $l_o=l_1$ and $m_o=m_1$, then $1/\tau_1$ decreases by slowing down l_{-o} to $l_{-o}\frac{K_2Y}{K_1X}$ at higher concentrations of reactants if $K_2>K_1$ ($X\approx Y$). Correspondingly $1/\tau_2$ decreases by slowing down m_{-o} to $m_{-o}\frac{K_3Z}{K_2Y}$ if $K_3>K_2$ ($Z\approx Y$). In this picture the equilibrium from E_1 goes over E_2 to E_3 when tRNA binds with increased affinity to E_2 and E_3 ($K_3>K_2>K_1$).

Although fast binding steps are seen at the higher concentrations investigated (Fig. 4c) the sensitivity in detection is not adequate for a detailed analysis of three additional fast relaxation times, which are expected to occur if the affinity of tRNA is different for the three forms E_1, E_2 and E_3. However, if the distribution of E_1, E_2 and E_3 is known, i.e. the values of L_o, M_o, L_1 and M_1 the individual rate constants

for the conformational transition can be calculated from the chemical relaxation data without knowledge of K_1, K_2, K_3 (17,18). As was shown in the case of tRNA covalently labelled with EB this can be accomplished by an analysis of the fluorescence lifetimes. Here each conformation is characterized by a characteristic lifetime of the fluorescent label and from the amplitudes of the exponential decay after pulsed excitation, the conformational distribution can be calculated (17,18). Evidently an analysis of this kind is called for even in the case of valyl-tRNA synthetase in order to give a detailed description of the mode of interaction.

It is not surprising that the experiment where the EB-$tRNA^{Val}$ was observed gives a picture very similar to that found when valyl-tRNA synthetase was observed, since, except for the lower temperature in the case of EB-$tRNA^{Val}$, the experiment was made under identical conditions. According to theory (22,23) the same spectrum of relaxation times should be observed and the data only show that the label is able to signal the conformational changes of the valyl-tRNA synthetase, as does the tryptophan of the synthetase. Since EB evidently is bound to $tRNA^{Val}$ even in the tRNA synthetase complex, it is near at hand to think about a change of the tRNA conformation concommitant with those found for the synthetase. From the work of Bittman (16) as well as from own data obtained on $tRNA^{Val}$ (Rigler, unpublished data) it is evident that three conformational transitions in the time range 30 sec^{-1}, 5 sec^{-1} and 0.5 sec^{-1} of the EB-tRNA complex can be observed in all tRNAs. Two of them are occurring at about 10 sec^{-1} and 2 sec^{-1} at Mg^{2+} concentration above 10 mM could be observed in $tRNA^{Phe}$ labelled with ethidium in position 16/17 or 37 as well as unlabelled yeast $tRNA^{Phe}$ (17,18). These conformations depend on the presence of Mg^{2+} and are not induced by ethidium. It is thus very likely that the data obtained from the relaxation of EB bound to

tRNA in a reversible manner show the relaxation of conformational states of tRNAs which are common to all tRNA, with EB as a very sensitive indicator.

Numerical values for the individual rate constants of the conformational transition can not be provided as yet. However, what is of primary importance, the existence of tRNA dependent conformational changes of valyl-tRNA synthetase can be safely concluded. It is of interest to point out that the rate constants obtained from the slower relaxation time under saturating conditions ($\sim$2 sec^{-1}) are in the same time range as the turnover number (2-4 mole (25) and may constitute a rate limiting step in the discriminatory and catalytic function of the enzyme.

Allosteric conformational changes have been postulated as a mechanism for selection of tRNA on the synthetase (1) and on the programmed ribosome (26) and have been deduced from the dependence of the rate of formation of aa-adenylate on the presence and absence of cognate tRNA (27,28).

It is here for the first time that conformational transitions of a synthetase in a time range in which aminoacylation of tRNA occurs, are observed. The possibility to observe these conformational transitions by the tRNA bound ethidium as indicator allows us to study a variety of tRNAs and their synthetases and to test the generality of the observations reported, as well as their significance for the discriminatory power of the enzyme.

ACKNOWLEDGEMENT

This study was supported by funds from the Swedish Cancer Society and the Swedish Natural Science Research Council.

TABLE I

Binding parameters for the interaction between $tRNA^{Val}$ and valyl-tRNA synthetase (VRS).

	T	n	K
$tRNA^{Val}$	21.5	1.2	$2.9 \times 10^{6} M^{-1}$
$tRNA^{Val}$-EB	21.3	1.3	$3.2 \times 10^{6} M^{-1}$

ΔH -2.6 Kcal $mole^{-1}$

ΔS $+20.4$ cal $mole^{-1} deg^{-1}$

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FIGURE CAPTIONS

Fig. 1. a) Titration of valyl-tRNA synthetase (VRS, $1x10^{-6}$M) with $tRNA^{Val}$ in phosphate buffer at 21.5°C. b) Scatchard plot of the data from Fig. 1a for the binding of $tRNA^{Val}$ to VRS. r is the number of moles of VRS bound per mole of tRNA. (VRS) is the concentration of free enzyme.

Fig.2. a) Titration of ethidium bromide (EB: $2x10^{-6}$M) with $tRNA^{Val}$ in phosphate buffer at 15°C. Anisotropy (r, ●) and intensity (I, ▲) of fluorescence were recorded. b) Scatchard plot of the data from Fig. 2a for the binding of EB to $tRNA^{Val}$. r is the number of moles EB bound per mole of tRNA.

Fig. 3. a) Titration of $tRNA^{Val}$-EB (EB: $2x10^{-6}$M, $tRNA^{Val}$ $2x10^{-6}$M, closed symbols) and of EB ($2x10^{-6}$M, open symbols) with VRS in phosphate buffer at 15°C. Anisotropy (r, ●, ○) and intensity of fluorescence (I, ▲, △) were recorded. b) Scatchard plot of the data from Fig. 3a for the binding of the $tRNA^{Val}$-EB complex to VRS. r is the number of moles $tRNA^{Val}$-EB bound per mole VRS.

Fig. 4. Time course of the fluorescence intensity of VRS in the presence of $tRNA^{Val}$ in phosphate buffer after a temperature jump from 25 to 32°C at different time scales (a,b;c,d).
a) and b) VRS $3x10^{-6}$M, $tRNA^{Val}$ $3x10^{-6}$M.
c) and d) VRS $1x10^{-5}$M, $tRNA^{Val}$ $1x10^{-5}$M.

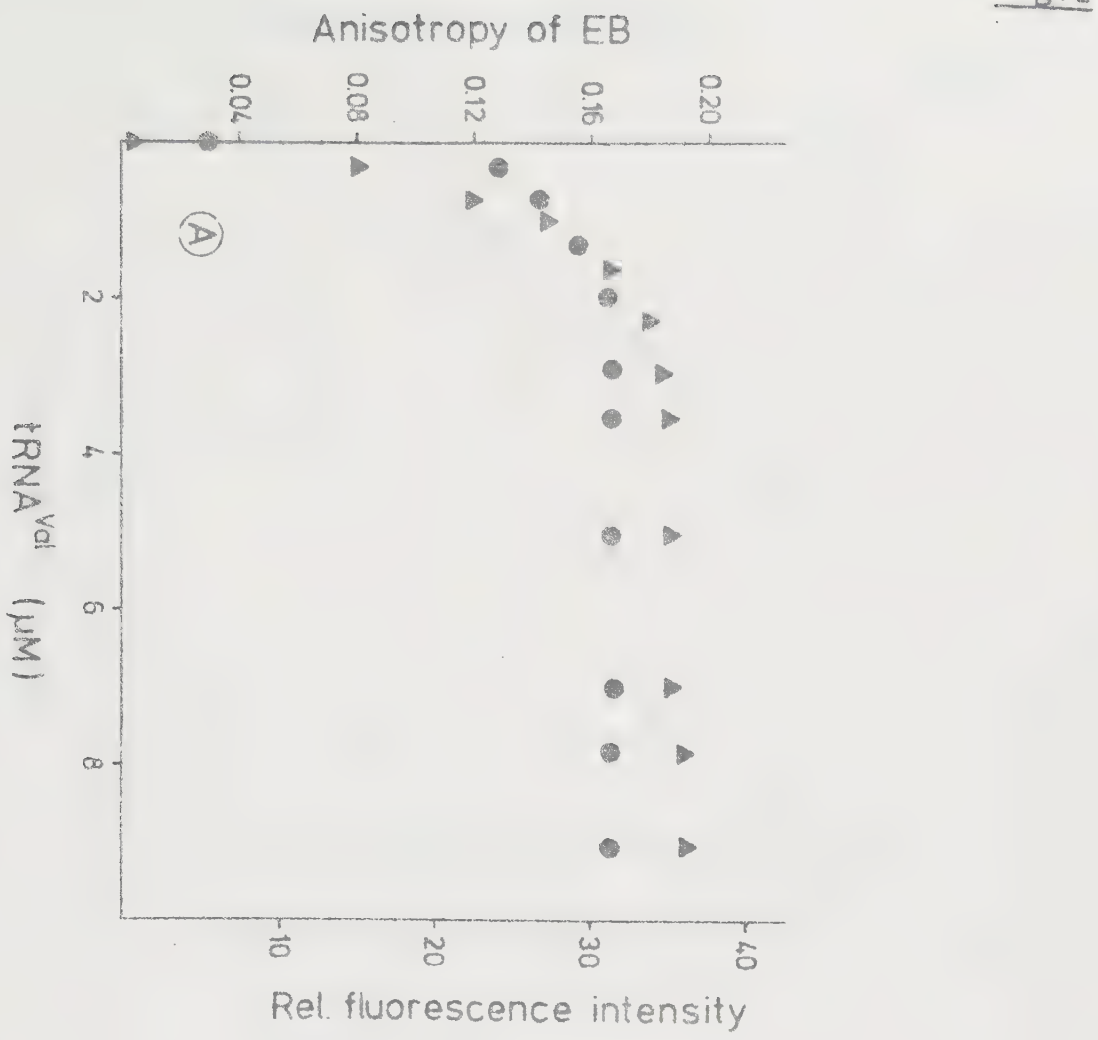
Fig. 5. Time course of the fluorescence intensity of the $tRNA^{Val}$-EB in the presence of VRS in phosphate buffer after a temperature jump from 15° to 18°C at different time scales (a,b). EB=$0.5x10^{-7}$M, $tRNA^{Val}$=$1.5x10^{-6}$M, VRS=$1.5x10^{-6}$M.

Fig. 6. Reciprocal relaxation times $1/\tau_1$ (a) and $1/\tau_2$ (b) as a function of the free concentration of $tRNA^{Val}$ and VRS. Closed symbols (●) values obtained from tryptophan fluorescence of VRS at 32°C. Open symbols values obtained from EB fluorescence of $tRNA^{Val}$ at 18°C. Half closed symbols values for VRS at 32°C (in absence of $tRNA^{Val}$).

Fig.1

Rel. fluorescence intensity
0.2
0.4
0.6
0.8
1.0
A
1
2
3
4
5
6
tRNA^Val (μM)
r/[VRS] [10^6 M^-1]
1
2
3
4
B
0.2
0.4
0.6
0.8
1.0
r

Fig. 2


Anisotropy of EB
0.04
0.08
0.12
0.16
0.20
Ⓐ
2
4
6
8
tRNA^Val (μM)
10
20
30
40
Rel. fluorescence intensity

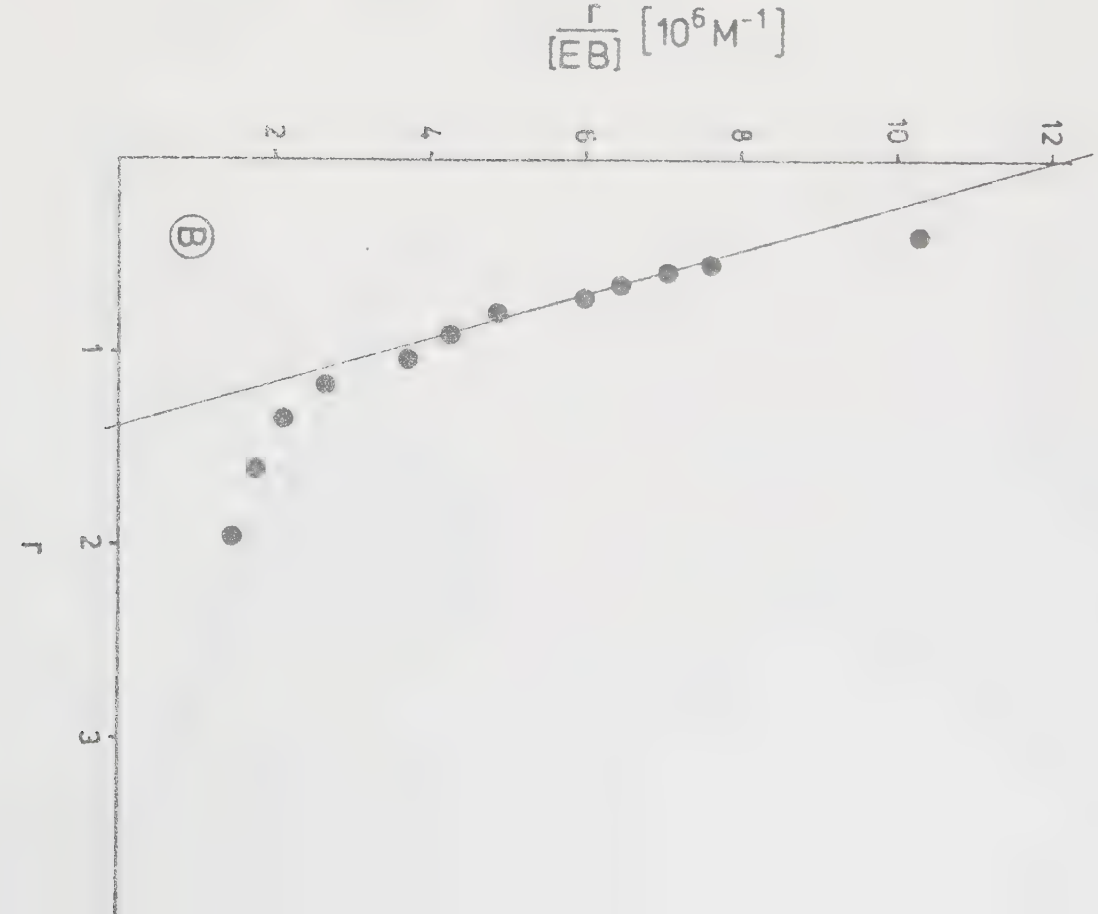

r/[EB] [10^6 M^-1]
2
4
6
8
10
12
Ⓑ
1
2
3
r

Fig. 3

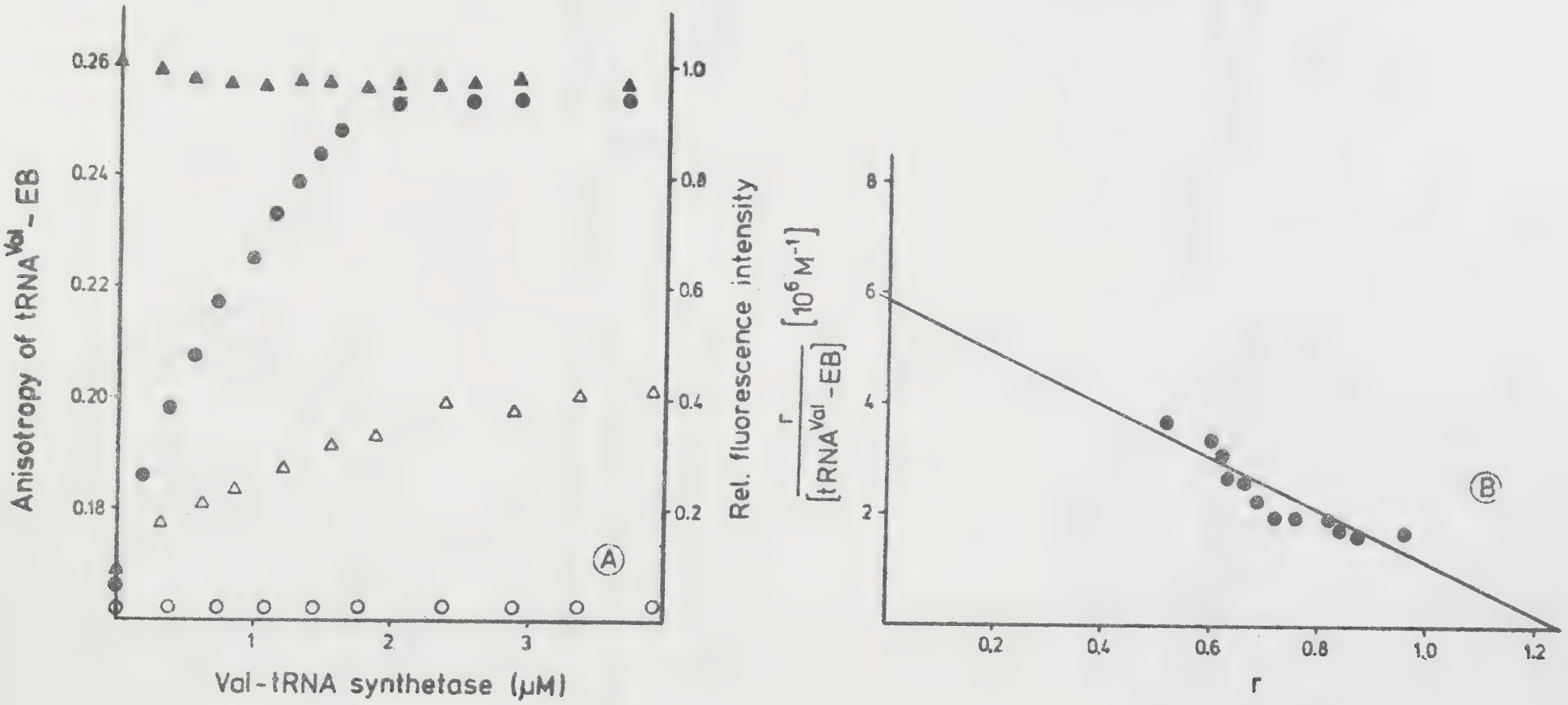

Anisotropy of tRNA^Val - EB
Rel. fluorescence intensity
Val-tRNA synthetase (μM)
0.26
0.24
0.22
0.20
0.18
1.0
0.8
0.6
0.4
0.2
1
2
3
A
B
r / [tRNA^Val - EB] [10^6 M^-1]
r
8
6
4
2
0.2
0.4
0.6
0.8
1.0
1.2

Fig. 4

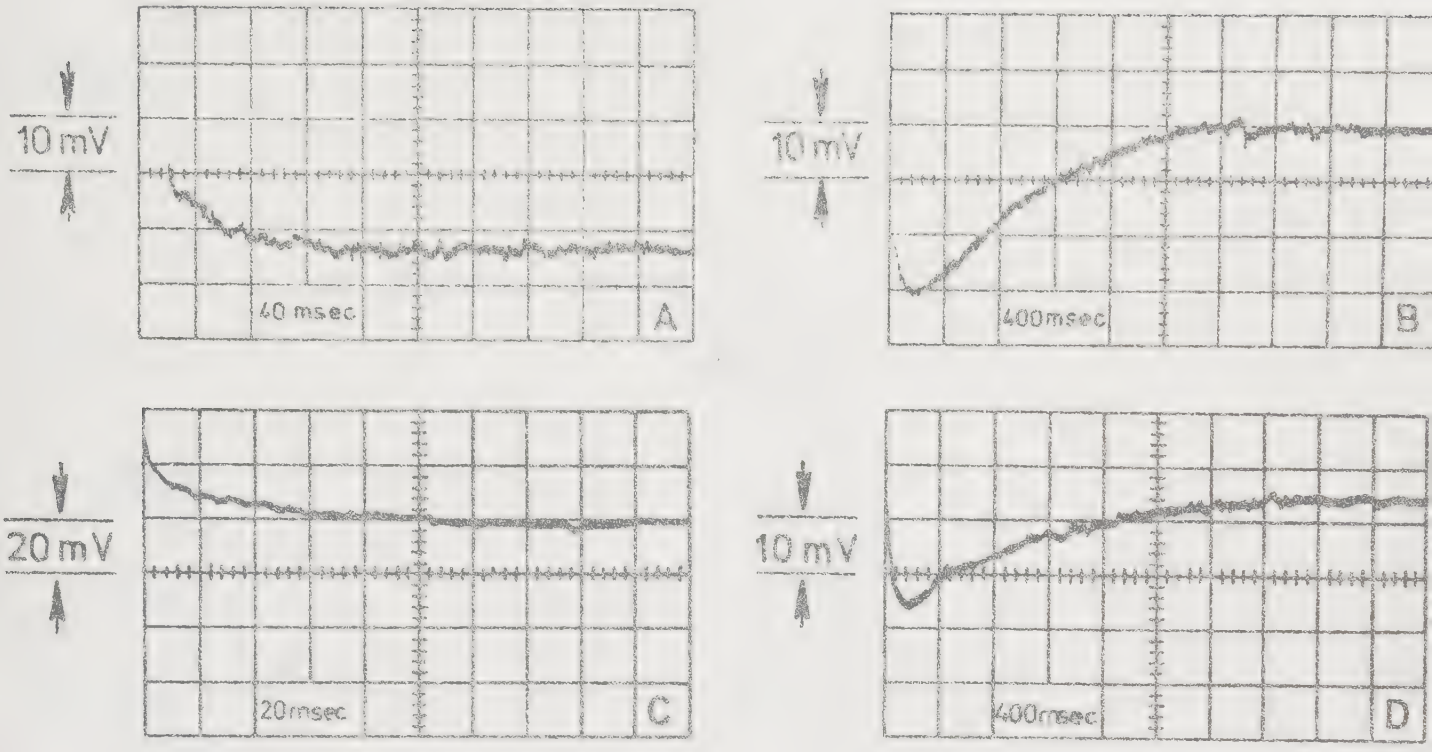

10 mV
40 msec
A
10 mV
400msec
B
20 mV
20msec
C
10 mV
400msec
D

Fig. 5

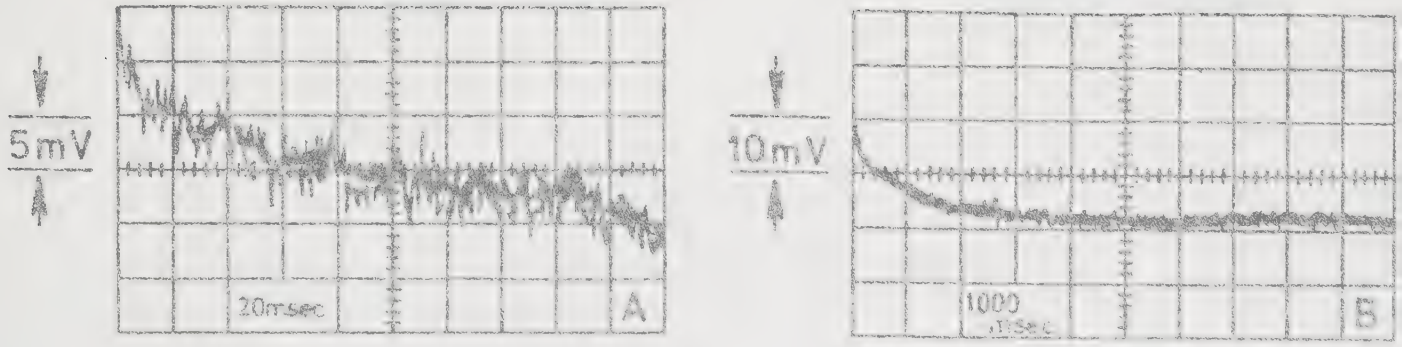
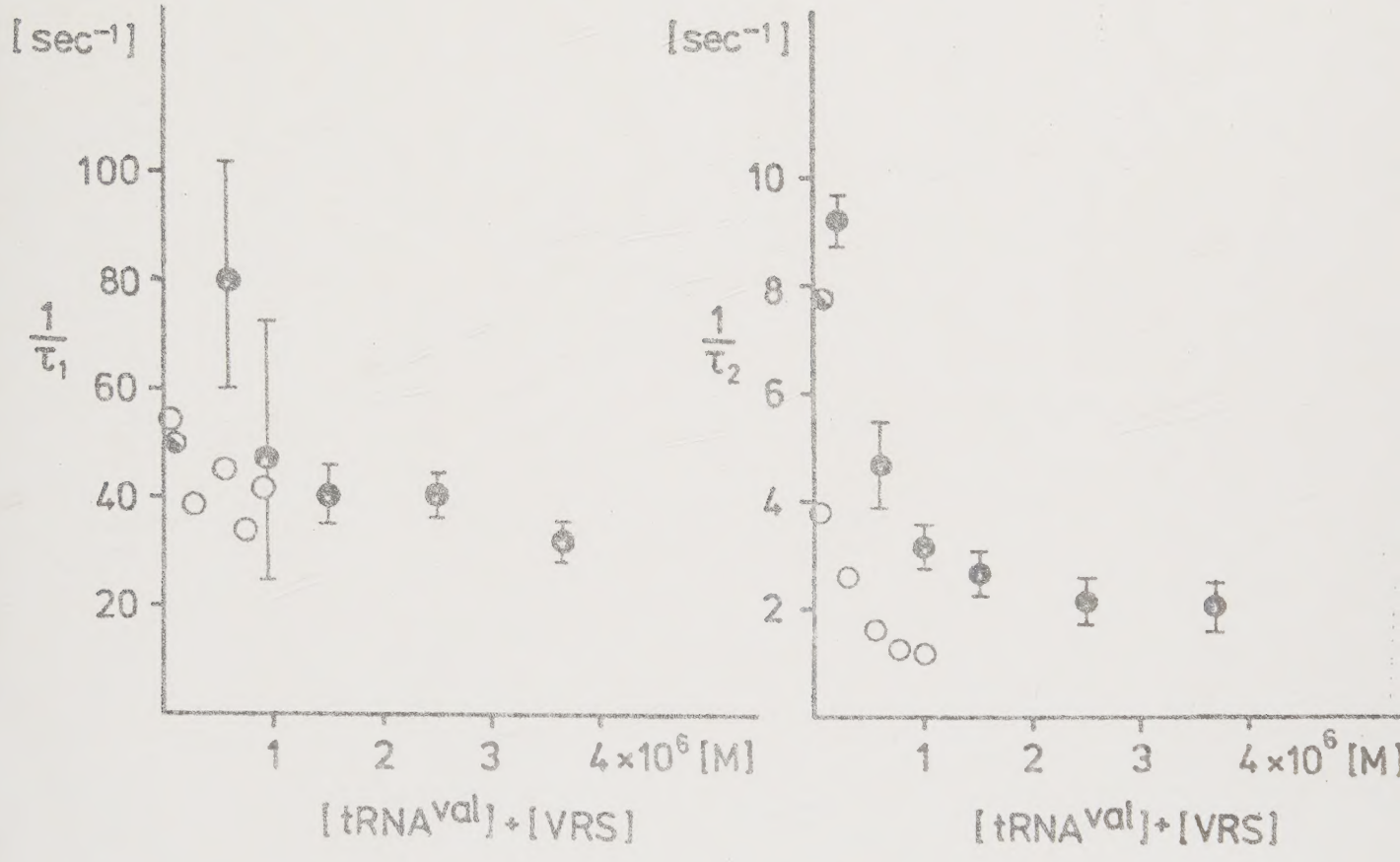

5mV
20msec
A
10mV
1000
B

Fig. 6


[sec⁻¹]
100
80
60
40
20
1/τ1
1
2
3
4×10⁶ [M]
[tRNA^val] + [VRS]
[sec⁻¹]
10
8
6
4
2
1/τ2
1
2
3
4×10⁶ [M]
[tRNA^val] + [VRS]